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Establishment of a non-human primate *Campylobacter* disease model for the pre-clinical evaluation of *Campylobacter* vaccine formulations

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Abstract

Campylobacter jejuni is a common cause of enteritis worldwide. The mechanisms by which *C. jejuni* causes disease are unclear. Challenge studies in humans are currently considered unethical due to the possibility of severe complications, such as Guillain–Barré syndrome. *Campylobacter* infection in non-human primates closely mimics the disease and immune response, seen in humans. In this study, we attempted to determine the minimal dose of a pathogenic *C. jejuni* 81-176 strain required for clinical signs and symptoms of disease ($\geq$80% attack rate) in *Macaca mulatta* monkeys using an escalating dosage (three doses for three monkey groups: 10^7, 10^9 and 10^{11} cfu). Eighty percent of the monkeys challenged with highest dose (10^{11} cfu) had mild disease, but the 80% attack rate (moderate diarrhea in 80% of the monkeys) was not achieved. However, 100% of monkeys showed IgA seroconversions (three-fold over pre-challenge titers). The elicited immune response was challenge dose-dependent. *Campylobacter* antigen specific fecal s-IgA responses were observed in all challenged groups but the response was not dose-dependent. Only IgM antibody secreting cells response was observed against *Campylobacter* antigens. The elicited immune response in three groups of rhesus monkeys was dose-dependent, indicating this monkey model can be used for pre-clinical evaluation of *Campylobacter* candidate vaccines, however these adult rhesus monkeys are less prone to *Campylobacter* infection.

Keywords: *Campylobacter* infection; Rhesus monkey; Immune response

1. Introduction

Campylobacter jejuni is the most common bacterial cause of food-borne disease in the United States and remains one of the most common bacterial causes of diarrhea worldwide [1,2]. In addition it causes a more severe illness than most other causes of travelers' diarrhea [3–6]. *C. jejuni* and *C. coli* infections are endemic worldwide and hyperendemic in developing countries. The epidemiology of *Campylobacter* infections is quite different in developing countries than in the industrialized world. In tropical developing countries, *Campylobacter* infections are hyperendemic among young children, especially those aged <2 years. Asymptomatic infections occur commonly in both children and adults, whereas, in developed countries, asymptomatic *Campylobacter* infections are unusual. Infection with this organism leads to a significant amount of acute illness as well as to occasional serious life-threatening acute neurological disease, the Guillain–Barré syndrome [7,8]. Most typically, infection with *C. jejuni* results in an acute, self-limited gastrointestinal illness characterized by diarrhea, fever, and abdominal cramps. Clinically, *Campylobacter* infection is indistinguishable from acute gastrointestinal infections produced by other bacterial pathogens, such as *Salmonella*, *Shigella*, and *Yersinia* species. Domestic and wild animals

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are the reservoirs for the organisms. Outbreaks are associated with contaminated animal products or water.

Even though infection with *C. jejuni* is more frequent than that with *Salmonella*, understanding of *C. jejuni* pathogenesis and the critical virulence factors needed is only just beginning to emerge. Although the mechanisms by which *C. jejuni* causes disease are unclear, the roles of motility, chemotaxis, and invasion have been partially established. A number of virulence-related properties of this important pathogen, including motility, attachment, colonization and invasion, have been described [9].

A good animal model is required to study the disease's progression and to evaluate candidate vaccines and drugs. The Rhesus macaque (*Macaca mulatta*) is a useful non-human primate species used in biomedical research. *Campylobacter* infection in non-human primates closely mimics the disease and immune response seen in humans and it is believed that such a model would be more predictive of human outcomes than other models. For example: (i) experimental infection in infant non-immune macaques elicits clinical signs of colitis with watery and occasionally bloody diarrhea [10–12]. Infant macaques appear to be resistant to diarrhea upon experimental homologous rechallenge and exhibit an anamnestic antibody response and a shorter duration of carriage (1–1.5 weeks); (ii) when young Rhesus monkeys (*M. mulatta*) were infected orally with a human strain of *C. jejuni* the disease induced was mild, characterized by a short duration of diarrhea and by a prolonged intermittent excretion of *C. jejuni* in the feces. Bacteraemia was generally present for 2–3 days and later the organisms localized in the liver and gall bladder. Recovered animals, when challenged with the same strain, showed no clinical symptoms or bacteremia, and excreted the organisms in the feces for only 3 days [13]. These data suggest that macaques are a good model for challenge study as well as testing for *Campylobacter* vaccine candidates.

In this study, we attempted to determine the minimal dose of pathogenic *C. jejuni* 81-176 strain required to establish clinical signs and symptoms of disease with a ≥80% attack rate (moderate diarrhea in 80% of the monkeys) in adult *M. mulatta* monkeys in response to escalating dosage levels and we measure immunogenicity. An advantage of using adults, rather that infant or young monkeys, is that one can collect sufficient blood to perform several immunological assays on multiple time points. Collection of blood and stool samples before and at multiple time point after experimental infection from the adult monkeys makes this an attractive animal model to study the kinetics of immune responses.

2. Materials and methods

2.1. Experimental design

To achieve an attack rate of moderate diarrhea in ≥80% of the monkeys, three groups of 10 monkeys each were sequentially challenged, starting with a dose of 10^7 cfu. In addition, a control group of five monkeys was included that were given saline. *C. jejuni* strain 81-176, the well characterized Lior serotype 5 strain that has been previously used in human challenge studies [14], was selected as the challenge organism. The optimal dose that we sought to choose was one that would result in ≥80% attack rate and clinical signs similar to human disease (such as loose stools, bloody diarrhea, fever, lethargy). Thus, the attack rate of Campylobacteriosis was based on the occurrence of moderate diarrhea.

The first group was challenged with a 10^7 dose of *C. jejuni* 81-176, and if after 14 days an 80% attack rate was not achieved the second group of 10 monkeys was challenged with a dose of 10^9 cfu. If after 14 days an 80% attack rate was still not achieved, the third group of 10 monkeys was to be challenged with a dose of 10^{11} cfu. The control group of five monkeys received normal saline.

A total of 35 adult *M. mulatta* were selected that were free of current *Campylobacter* infection (as determined by negative fecal cultures obtained 21 and 3 days prior to challenge) and had *Campylobacter*-specific serum ELISA IgA & IgG titers <1:100. The male and female monkeys between 4 and 10 years of age, weighing >5.5 kg, came from the Rhesus monkey breeding colony at the Department of Veterinary Medicine, AFRIMS. All monkeys were tuberculosis negative, Simian Immunodeficiency Virus negative, Simian Retrovirus negative and Simian T-Lymphotropic Virus negative. Only monkeys in good health as shown by physical examination were used.

2.2. Challenge study

After screening, the selected monkeys were moved from colony cages to individual cages 28 days before the challenge date. Monkeys were fasted overnight before challenge and 90 min after challenge. Monkeys were given the *C. jejuni* 81-176 challenge inoculum in 10 ml of PBS. Monkeys were anesthetized with ketamine hydrochloride (10–20 mg/kg) given intramuscularly in the caudal thigh before nasogastric administration of the inoculum. A number 8 French pediatric feeding tube 15 in. long was used. Gastric fluid was aspirated with a sterile syringe to ensure the correct positioning in the stomach. Twenty milliliters of (1.33%, pH 8.12) sodium bicarbonate solution was administered intragastrically through the stomach tube. Ten minutes later this procedure was repeated and gastric contents were aspirated to measure pH (expected value 7 or higher) before administration of the inoculum. The 10 ml of inoculum were then administered by stomach tube. The five sham control monkeys were given 10 ml of normal saline. After challenge all monkeys were under close observation.

2.3. Inoculums preparation

A working seed of *C. jejuni* 81-176 was used to grow the strain according to the method of Black et al. [14]. The experiments were conducted according to the principles set

forth in reference [15]. Strain 81-176 was grown on Tryptic soy agar plates supplemented with 5% sheep red blood cells (sBAP) (Remel) in a microaerobic atmosphere (5% O_2, 10% CO_2, and 85% N_2) at 42–43°C for 22–24 h. Approximately 150–300 well isolated colonies were harvested into Thioglycollate broth suspension medium (Remel) and inoculated onto Mueller-Hinton soft agar (MHSA) plates and incubated in microaerobic atmosphere at 42–43°C for 20–24 h. Highly motile *Campylobacter* from MHSA plates were selected and harvested into Thioglycollate broth suspension medium. The suspension was adjusted and inoculated onto *Brucella* agar plates supplemented with 5% sheep blood, followed by incubation in microaerobic atmosphere at 42–43°C for 18–22 h. The cultures were harvested with PBS and used to initiate infection in monkeys, similar to as it was done for mice in other studies [16,17]. Initially, the number of *Campylobacter* cells in the suspension was estimated spectrophotometrically; the challenge dose was subsequently verified by plating serial dilutions of the inoculum onto sBAP.

2.4. Fecal excretion and bacteriology

Rectal swabs and fecal samples from each monkey were cultured twice (am and pm) daily. Fecal samples were cultured on *Brucella* agar plates supplemented with 5% sheep blood by filtration technique and modified-charcoal cefoperazone deoxycholate agar, followed by incubation in microaerobic atmosphere at 42–43°C. After 48 h of incubation, suspect *Campylobacter* colonies were confirmed by morphology, catalase, oxidase, hippurate hydrolysis reactions, susceptibility to cephalothin antibiotic discs (CF 30 μg), and nalidixic acid antibiotic discs (NA 30 μg), slide agglutination with Lior 5 antiserum. A monkey was considered negative if no *C. jejuni* colonies were detected in fecal sample on three consecutive days. Fecal samples were also used to inoculate MacConkey, Hektoen Entric, and thiosulfate-citrate-bile salt sucrose agar to screen for other bacterial enteropathogens.

2.5. Stool characteristics

Stool samples were described as being normal, loose, or watery, and were scored as shown in Table 1. The presence of mucus and/or blood was also assessed and scored (Table 1). Monkeys passing a single stool with gross blood were considered positive for dysentery, and animals passing diarrheal stools on two consecutive days were considered positive for diarrhea.

2.6. Illness index/monitoring of infection

An index was developed by the Veterinary-Medicine Department of AFRIMS to allow quantitative expression of the relative degree of morbidity and mortality associated with the challenge dose, as adopted from the "Guidelines on the recognition of pain" [18]. Following challenge, animals were monitored three times daily for signs of diarrhea, dehydration, appetite, and water consumption. Monkeys were observed for 14 consecutive days and assigned a daily numerical score (Table 1). The group means of these daily indices are presented as the illness indices of various challenge groups.

Grading of diarrhea in monkey:

Stool grading:

- Grade 0—hard (normal)
- Grade 1—soft

Table 1
Scoring system of clinical symptoms after oral inoculation of rhesus monkeys with *C. jejuni* 81-176 strain

ID. no.	Activity	Appetite	Stool consistency	Mucous in stool	Blood in stool	Dehydration status	Weight (kg)[a]	Weight change (%)	Temperature (°C)	Temperature change (°C)	Total score
Codes:											
Activity	0 = alert or move around; 1 = locomotion after slight stimulation; 2 = weakness, move slowly after moderate stimulation; 3 = sit quietly or recumbency, very depressed or obvious lethargy after stimulation										
Appetite	0 = ate all 7–10 chows; 1 = ate 3–6 chows; 2 = ate 1–3 chows; 3 = ate 0 biscuit										
Stool consistency	0 = normal; 1 = loose; 2 = watery										
Mucous in stool	0 = none; 1 = mucous is observed										
Blood in stool	0 = none; 1 = fresh blood is observed; 2 = frank blood										
Dehydration status	0 = normal well-groomed haircoat; 1 = slight dehydration, ungroomed haircoat, slightly reduced skin elasticity; 2 = moderate dehydration and thigh skin fold returns slowly; 3 = severe dehydration, sunken eye, and thigh skin fold stays										
Weight change[a]	0 = none; 1 = <10%; 2 = 10–15%; 3 = >15%										
Temperature change (°C)	0 = none; 1 = <1 °C; 2 = 1–2 °C; 3 = >2 °C										
Scoring:											
0–4	Normal										
5–9	Monitor carefully, should consider the use of analgesics										
10–14	Evidence of distress; should be regularly monitored; seek veterinary advise; consider initiating treatment										
15–20	Evidence of severe pain, analgesics must be given, seek veterinary advise; initiate treatment										

[a] Weight will be measured only when the animal has been anesthetized.

- Grade 2—loose
- Grade 3—watery

Diarrhea: To qualify as having diarrhea, a monkey had to pass at least two grade 3 stools within 48 h.

- *Mild diarrhea*: Soft to loose stool without blood or mucus; spontaneous recovery.
- *Moderate diarrhea*: Loose stool or mixed soft and loose stool with/or without blood or/and mucus. Dehydration (mild to moderate) requiring oral or parenteral electrolyte replacement. Mild depression, activity reduced, but still able to function. Transiently or moderately reduced appetite. Supportive treatment and specific antibacterial treatment required to maintain health status.
- *Severe diarrhea*: Frequent loose or projectile watery stool with blood or/and mucus in stool. Rapid dehydration (moderate to severe). Moderate to marked lethargy. Marked anorexia. Requires IV electrolyte and supportive therapy, and prompt antibacterial treatment, intensive care and close monitoring.

2.7. Temperature monitoring

Remote sensing transponder IPTT-200 (Bio Medic Data System Inc.) thermometers were placed on the monkeys subcutaneously on day 21 (after screening). Temperature was measured and recorded twice per day (Table 1).

2.8. Treatment

At day 14 post challenge, all monkeys were treated with a single oral dose of azithromycin, 20 mg/kg.

2.9. Blood samples

Monkeys were sedated with Ketamine 10–15 mg/kg IM, prior to collecting blood samples from a superficial vein; the blood was put into heparinized tubes. CBC, hematocrit, and MCV were monitored when necessary to ensure the well being of the monkeys. Plasma was separated and peripheral blood mononuclear cells (PBMC) were collected using histopaque-1077 (Sigma).

2.10. Antigens for immunological assays (ELISA and ELISPOT assays)

Immune responses were evaluated against the following antigens: formalin fixed *C. jejuni* strain 81-176 whole cells (F-CJ 81-176) and a glycine extract of outer membrane proteins that possesses multiple proteins [19] from *C. jejuni* strain 81-176.

2.11. Humoral immune responses

Systemic humoral immune responses in plasma was evaluated using a standard ELISA to detect IgA and IgG antibodies [20]. The IgA response is associated with acute infection. The IgG response is generally longer-lived [21].

2.12. Mucosal immune responses

Total and *Campylobacter*-specific mucosal responses were determined by measuring IgA, IgG, and IgM secreting cells in circulation using ELISPOT assay [22]. Mucosal total secretory immunoglobulin A (s-IgA) and *Campylobacter*-specific response was determined in fecal extracts using ELISA.

2.13. ELISA

Serum antibodies to *Campylobacter* antigens were measured by ELISA. Briefly, 96-well polystyrene microtiter plates (Nunc-Immunoplates, NUNC ELISA plate, Maxisorp) were incubated with coating buffer (0.10 M carbonate buffer, pH 9.6, 100 μl/well) containing: glycine extract (3 μg/ml), *C. jejuni* 81-176 formalin fixed whole cells (2×10^9 ml^{-1}) and bovine serum albumin (BSA) as control antigen (10.0 μg/ml) for 2 h at 37 °C followed by overnight at 4 °C [23]. After removal of the coating solution, the plates were washed three times with PBS pH 7.4 containing 0.1% Tween 20 (PBST) and then blocked with 3% BSA in PBST for 1½ h at 37 °C. The plates were then washed 3× in PBST. Serum were added to the wells in two-fold dilutions using 1% BSA in PBST and the plates were incubated for 3 h at 37 °C. After four further washings, peroxidase labeled goat anti-human: IgA and IgG secondary antibodies diluted in 1% BSA in PBST were added to the plates. The plates were then incubated for 2 h at 37 °C and were washed four times, and ELISA was completed by the addition of enzyme-substrate solution containing ABTS peroxidase (ABTS: 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) for 30 min at 22 °C. The OD was read at a wavelength of 405 nm with an ELISA plate reader, Spectra MAX 190, Molecular Devices. The endpoint titer was the highest dilution giving a net OD 405 of 0.15 (net OD is equal to antigen-specific OD – BSA OD). Data are presented as the geometric mean titer of the group. Positive and negative control sera were included in each plate in each of the assays.

The mucosal total s-IgA and *Campylobacter* antigen-specific responses were determined in fecal extracts using ELISA. The serum ELISA protocol was followed for each step except the secondary antibody was peroxidase labeled goat anti-human s-IgA. For total s-IgA, ELISA plates were coated with affinity purified goat-anti-human Ig, $F(ab')_2$, (Jackson ImmunoResearch Lab Inc), at 1.0 μg/ml in PBS. IgA Standard: Human IgA-secretory, highly purified (Fitzgerald) was used as IgA standard at 40 ng/ml conc. Amount of IgA in samples was determined by using human IgA curve standard, using SOFTmax PRO 4.0 software. *Campylobacter*-enteritis from other enteric infections can be distinguished by the *Campylobacter*-specific s-IgA responses in stool.

2.14. Fecal extraction procedure

Immediately after collection, stool samples were either treated with the extraction solution or were placed at −70 °C until extracted. For each 1 g of solid stool, 2 ml of extraction buffer (EB) (chilled PBS containing 1 mg/ml ethylenediamine tetra-acetic acid, 0.2 mg/ml trypsin soybean inhibitor, 1 mg/ml BSA, 17.5 mg/ml phenyl methyl sulfonylfluoride, and 0.05% Tween-20) were added; for each 1 g of semi solid stool 1 ml of EB was added; for each 1 ml of liquid stool, 0.1 ml of EB was added. The fecal sample with the EB was mixed (vortex at maximum speed for 5 min) and then filtered through a single layer of gauze (if necessary). The sample was centrifuged at 20,000 × *g* for 30 min at 4 °C and the supernatant collected, aliquoted, and stored at −70 °C until assayed.

2.15. Antibody secreting cells (ASC) assay

Antibody secreting cell assays were performed on fresh peripheral blood mononuclear cell specimens by ELISPOT assay. Briefly, Multiscreen Immobilon-P filtration, 96-well plates were incubated with coating buffer (0.10 M carbonate buffer (pH 9.6; 100 μl/well) containing: capture antibodies (goat anti-human IgA (G α IgA), G α IgG, and G α IgM), GE, F-CJ 81-176 and BSA as control antigen for 1 h at 37 °C followed by overnight at 4 °C in a humid chamber. The next morning, coating buffer from the plate was discarded and the plates were washed thrice with sterile PBS. The wells were blocked with 150 μl/well with blocking-media (5% newborn calf serum (NBCS) in RPMI 1640 media) for 1 h at 37 °C incubator.

After 1 h the RPMI medium wase discarded and complete RPMI medium (RPMI 1640 with 10% NBCS, 2 mM L-glutamine, 50 mg/ml gentamycin, filtered through 0.22 μm) 50 μl/well was added to keep the membrane moist. Fresh PBMCs from heparinized whole blood were adjusted to 10×10^6/ml in complete RPMI 1640, 50 μl of cell suspension was dispensed in each well, i.e., 5×10^5 cells/well. For total IgA, IgG, and IgM secreting cell assays, both 5×10^4 and 5×10^5 cells/well were seeded. Triplicate wells were used for each antigen. The plates were incubated overnight at 37 °C in 5% CO_2. The next morning, the contents of the wells were discarded and washed 4× with PBS-T (on shaker 2–3 min/wash). The final wash was with PBS only before adding conjugates. Alkaline phosphatase conjugated antibodies (A, G, and M) at 100 μl/well were dispended to the appropriate wells and incubated at 37 °C for 3 h. The plates were washed 4× with PBS-T and PBS as above and 1× with filtered TBS (last wash). Freshly prepared substrate was used (NBT/BCIP 1 Tab/10 ml $_dH_2O$, filter 0.2 μm and stored away from light). Working NBT/BCIP substrate at 50 μl per well was dispensed to appropriate wells. The plates were covered with aluminum foil to protect from light and incubated at room temperature for 15–30 min until spots formed. Then the plates were washed 5× with distilled water and left in water for 1 h or overnight to get rid of non-specific background. After the final aspiration, wells were blotted on absorbent paper to remove residual liquid. The plates were air-dried overnight, protected from light. Plates were scanned on a CTL Immuno Spot Analyzer to count the spots.

2.16. Data analysis

Attack rates of Campylobacteriosis in each group of monkeys were calculated based on the occurrence of diarrhea (two consecutive days) or dysenteric stools (one occurrence), representing "moderate diarrhea", in at least 80% of the monkeys in a group. A positive ELISA antibody response was defined as three-fold rise over pre-challenge titer. Seroconversion for IgA antibodies is ≥3 IgA titer on day 7/day 0. Similarly, seroconversion for IgG antibodies is ≥3 IgG titer on day 14/day 0. Data were analyzed by analysis of variance (ANOVA) for repeated measurements. p values of <0.05 are considered significant. Statistical analysis was performed using SPSS software packages.

3. Results

3.1. Clinical signs summary

Mild transient anorexia (Grades 1–2) is commonly observed in animals from this colony when the monkeys are disturbed, handled or anesthetized. Similarly, a slightly increased body temperature of 1–2 °F is not considered significant.

All monkeys in control group were generally active and appeared healthy during the study period. No control monkey exhibited diarrhea or significant clinical signs.

Of the first group of monkeys challenged with CJ 81-176 at 10^7 cfu, nine of the 10 (90%) were generally active and appeared healthy during the study period. Two monkeys (20%) passed soft stools. One monkey (10%) had moderate diarrhea consisting of transient soft or loose stools, sometimes with blood. A slight weight loss and mild to moderate dehydration were also detected in this animal. Analgesic and supportive electrolyte therapy were provided until this monkey returned to normal. An increased body temperature (2.2–2.7 °F) was recorded in another monkey. Overall, at this dosage level, an attack rate ≥80% attack rate was not achieved.

The second group of 10 monkeys was challenged with 10^9 cfu of *C. jejuni* 81-176. All these monkeys were generally active and appeared healthy during the study period. Eight monkeys (80%) passed transient soft stools or soft stool and normal stool for one to four times and spontaneously recovered. Increased body temperature (2.6–2.8 F) was recorded in one of these eight monkeys. Thus, neither was an attack rate ≥80% for moderate diarrhea achieved among the monkeys challenged with 10^9 cfu.

Finally, the third group of 10 monkeys was challenged with 10^{11} cfu. These monkeys were also generally active and

Table 2
Total score after evaluation of clinical symptoms, after oral inoculation of rhesus monkeys with *Campylobacter jejuni* 81-176 strain into three groups or normal saline into control group

Clinical observation days	Obtained total score (average) after clinical evaluation			
	Group C ($n=5$)	Group 1 ($n=10$)	Group 2 ($n=10$)	Group 3 ($n=10$)
Day (−3)	1.5	1.65	1.5	0
Day (−2)	0.2	0.6	0.15	0.1
Day (−1)	0.4	0.45	0.15	0.25
Day 0 (challenge day)	0.1	0.4	0.4	0.2
Day 1	0.1	0.3	0.45	0.6
Day 2	0.1	0.33	0.25	0.6
Day 3	0.1	0.9	0.45	0.6
Day 4	0.1	0.8	0.35	0.3
Day 5	0.1	0.55	0.25	0.25
Day 6	0	0.25	0.5	0.35
Day 7	1.6	1.65	0.8	0.4
Day 8	0.2	0.75	0.15	0.3
Day 9	0.3	1	0.2	0.4
Day 10	0.1	0.6	0.1	0.3
Day 11	0.1	0.4	0.05	0.25
Day 12	0.1	0.55	0	0.25
Day 13	0	0.2	0.15	0.2
Day 14	0.6	0.95	1.15	0.65
Day 15	0	0.45	1.25	0.3
Day 16	0.1	0.35	0.4	0.2
Day 17	0.1	0.2	0.45	0.1
Day 18	0.1	0.2	0.05	0.1
Day 19	0	0.2	0.1	0.1
Day 20	0	0.25	0.15	0.1
Day 28	1.3	1.15	0.65	0.3

healthy during the study period. Seven monkeys in this group passed transient soft stools or soft stool and normal stool on 1–6 occasions and spontaneously recovered. Only one monkey had loose stool for one day and blood in its stools for 3 days. None of the monkeys in this group scored >4 in clinical signs. Thus, at the peak dosage level of 10^{11} cfu of *C. jejuni*, an attack rate for moderate diarrhea ≥80% was not achieved.

The total clinical score for each monkey on each day was calculated. The average total clinical score for each group of monkeys is shown in Table 2. Only one monkey in group 1 had score of 5 for 2 days; this monkey had loose stools with mucus and loss of skin turgor on day 3 and day 4. Although most of the monkeys in each challenge group manifested a mild disease consisting of transient soft stools with a small amount of blood and mucus, in no challenge group did 80% of the monkeys get moderate diarrhea and the observed clinical signs categorized the monkeys as "normal". Therefore, the 80% attack rate was not achieved in any of the challenged group.

3.2. Shedding of CJ 81-176

None of the five control monkeys shed *C. jejuni*. In Table 3, shedding of the challenge strain is shown as a percentage of the total monkeys in each group. There is no significant difference among three groups. Following treatment with antibiotic on day 14, shedding of CJ 81-176 was abruptly curtailed. No other pathogenic bacteria were detected in any fecal samples from the 35 monkeys.

3.3. Serum IgA and IgG titer evaluation

There was no significant increase in IgA titers against *Campylobacter* antigens in the control group during the study period. IgA titers against formalin fixed *C. jejuni* 81-176 and GE antigen in the four groups of monkeys are shown in Fig. 1. In group 1 (challenged with 10^7 cfu), the highest IgA titers were observed on day 14, whereas, peak titers for group 2 were observed on day 7. Responses of monkeys in group 3 (challenged with 10^{11} cfu) are also shown in Fig. 1A and B. In general, IgA titers against the GE antigen are higher compared to titers measured using the F-CJ 81-176 antigen. The IgA titer against both antigens is higher in group 2, but there was a large variation in titer values among 10 monkeys in group 2, which is indicated with a high standard deviation.

All three challenged groups, IgG titer is peaked on day 14 (Fig. 1C and D). The IgG responses exhibited a dose response when measured against F-CJ 81-176 antigen; against GE the titer in group 3 is higher compared to group 2 titers except on day 14. On day 14, the IgG titer value in group 3 is significantly higher compared to IgG titers in group 2. Similarly, the titer in group 2 is significantly higher compared to group 1 IgG titers.

The mean IgA titer against both antigens was higher in group 2 monkeys, whereas the IgG response against both antigens was higher in group 3 monkeys. The specific responses of monkeys with IgA and IgG seroconversions, are shown in Table 4.

3.4. Fecal total s-IgA and antigen-specific s-IgA

Fecal total s-IgA was measured by ELISA with an IgA standard curve as shown in Fig. 2A. The level of total s-IgA in fecal samples in each group is not homogeneous. The level

Table 3
Shedding of challenge strain *C. jejuni* 81-176 from monkeys in three groups on different days, result expressed as percentage

Monkey group	Day 0 (challenge day)	Day 1	Day 2	Day 3	Day 5	Day 7	Day 9	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16
Group 1 (10^7 cfu)	0	70	80	80	90	100	80	70	60	50	30	0	0
Group 2 (10^9 cfu)	0	100	100	100	90	90	80	50	60	20	40	0	0
Group 3 (10^{11} cfu)	0	90	100	100	90	100	70	50	80	70	60	0	0

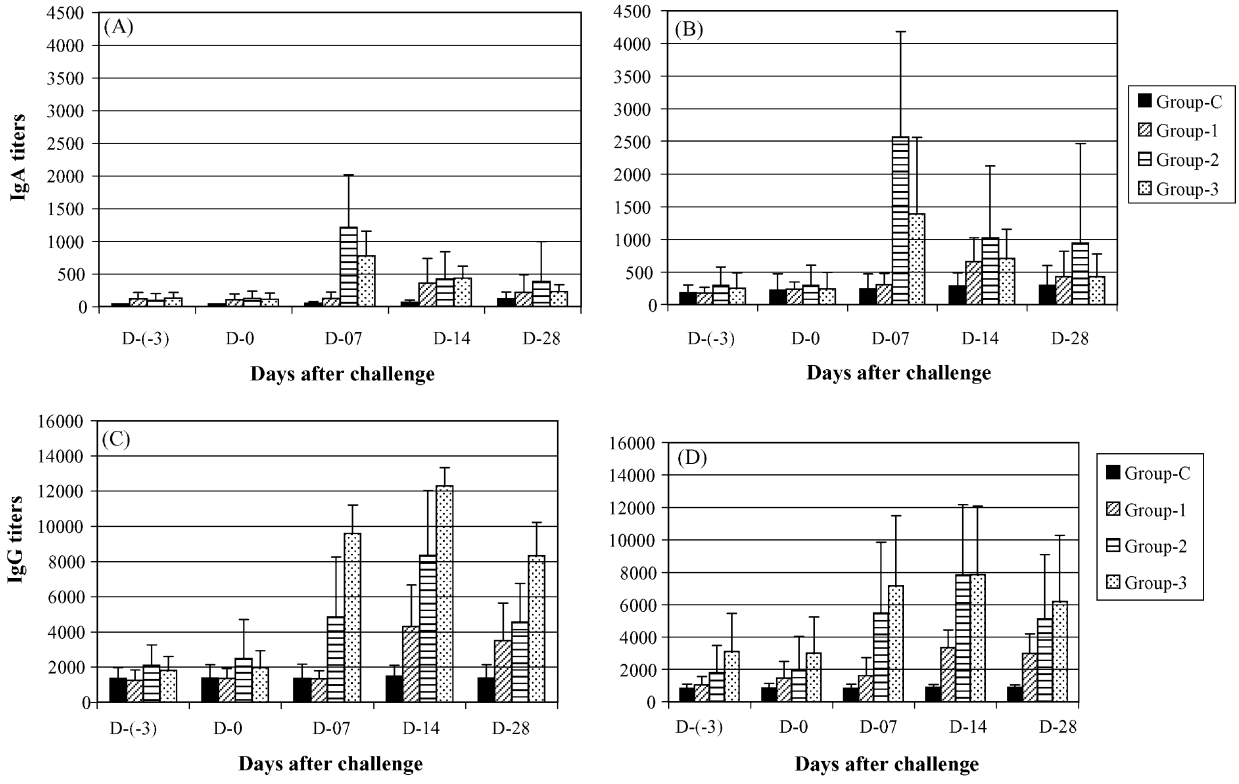

Fig. 1. Plasma IgA and IgG antibody titers in control group and three challenged monkey groups are shown as mean value with standard deviation bar, strain (A). IgA titers: against antigen F-CJ 81-176 strain (A), against antigen GE (B), IgG titers: against antigen F-CJ 81-176 strain (C), against antigen GE (D). Monkeys were challenged on day 0 (D-0). Day 0 samples were collected before challenge. Blood were collected on day 3 prior to challenge (D-(−3)), challenge day (D-0), day 7(D-7), day 14 (D-14) and on day 28 (D-28) to monitor elicited immune response.

Table 4
Number of sero-converted monkeys in control and three different challenged groups

Group	Number of monkeys							
	IgA titer				IgG titer			
	D07/D0 IgA (F-CJ)	D14/D0 IgA (F-CJ)	D07/D0 IgA (GE)	D14/D0 IgA (GE)	D14/D0 IgG (F-CJ)	D28/D0 IgG (F-CJ)	D14/D0 IgG (GE)	D28/D0 IgG (GE)
Control	0	0	0	0	0	0	0	0
1 (10^7 cfu)	0	6	0	4	5	4	5	2
2 (10^9 cfu)	7	7	7	5	8	3	6	5
3 (10^{11} cfu)	10	8	9	8	8	6	7	4

of total s-IgA on day 2 and day 3 was highest in group 3 and on day 4 and day 5 was highest in group 1. The maximum, median, and minimum levels of total s-IgA in fecal samples are shown in Table 5. The antigen-specific s-IgA in fecal samples is expressed as titer/100 ng of total s-IgA, which is shown in Fig. 2B and C.

Table 5
The maximum, median, and minimum levels of fecal total s-IgA (ng/ml) in control monkey group and three challenged monkey groups

Values	Group C	Group 1	Group 2	Group 3
Maximum	870	1126 (day 4)	520 (day 2)	1204 (day 2)
Median	508	606	366	429
Minimum	243	230 (day 28)	190 (day 28)	95 (day 28)

The presence of antigen-specific s-IgA in fecal samples was not consistent (Fig. 2B against antigen F-CJ 81-176 and Fig. 2C against antigen GE). Among monkeys' in-group 1, one had no antigen-specific s-IgA in stool samples. Three to four monkeys had both antigen-specific s-IgA responses only on 2 days.

In group 2, three monkeys did not show any antigen-specific s-IgA response and two monkeys showed rises only on 2 days. The antigen specific titer/100 ng s-IgA is higher in group 2 compared to group 1 and group 3. Among group 3, one or two monkeys showed no s-IgA response against the *C. jejuni* antigens; three monkeys showed rises only on 2 days against F-CJ 81-176 and one monkey showed s-IgA response on 2 days against GE.

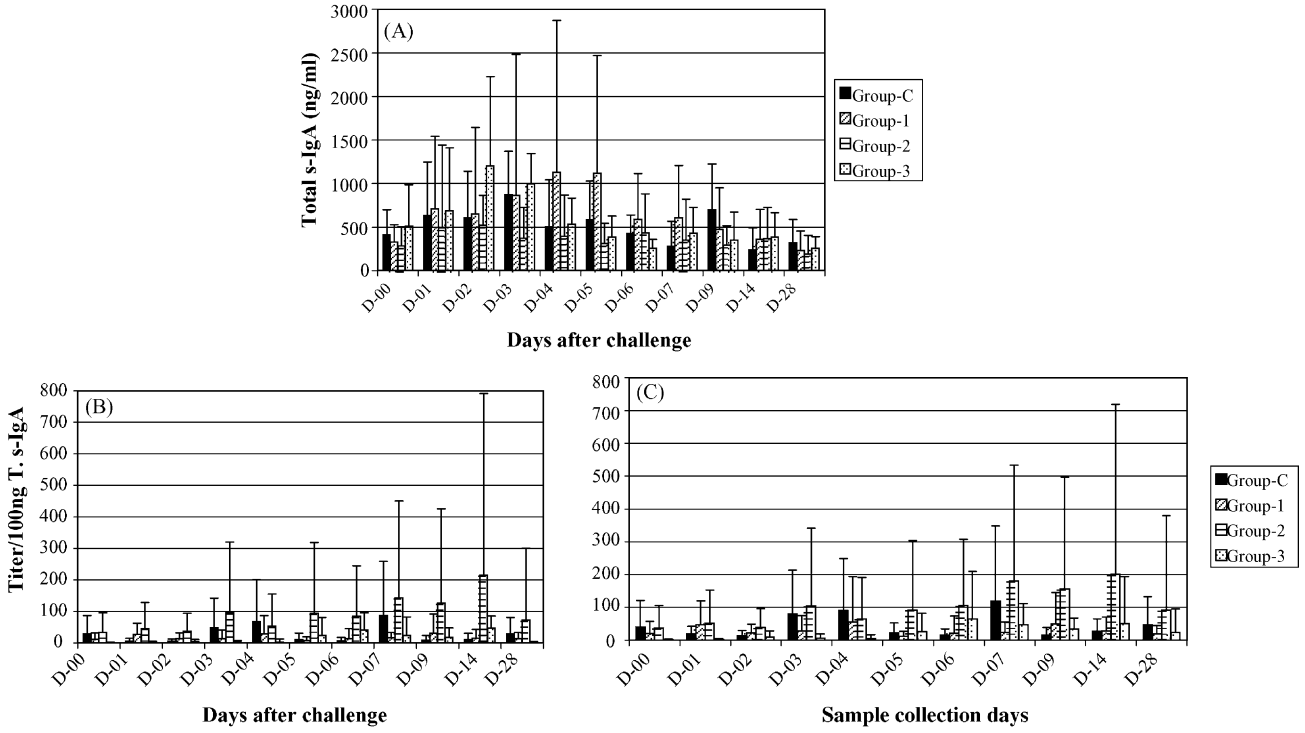

Fig. 2. Mean fecal total s-IgA (ng/ml) (A) and antigen-specific s-IgA in control monkey group and three challenged monkey groups. Antigen-specific s-IgA is expressed as IgA titer/100 ng total s-IgA. s-IgA titer against F-CJ 81-176 is shown in (B), and against GE in (C)

There was no correlation between antigen-specific s-IgA and total s-IgA. In addition, there was no correlation between challenge dose and total s-IgA or antigen-specific s-IgA in fecal samples.

Notably, the one monkey in group 1 that exhibited a clinical score of 5 showed the highest antigen-specific s-IgA in fecal samples compared to the other nine monkeys in group 1. However, monkeys in group 3 that had mucus and blood in their stool had antigen-specific s-IgA only on 2 days. There was no correlation between clinical severity and antigen-specific fecal s-IgA responses. There was also no correlation between plasma IgA or IgG response with fecal antigen-specific s-IgA response in any group.

3.5. ASC response by ELISPOT assay

Total IgA, IgG, and IgM ASC responses in the control group and the three challenged groups are shown in Fig. 3(A–C). Antigen specific ASC response were very meager overall. The antigen-specific IgA and IgG response was almost nil. Only antigen-specific IgM ASC response were seen, which was highest in group 2 monkeys, as shown in Fig. 3(D and E).

4. Discussion

An intragastric animal model is useful for research involving pathogenesis of bacterial enteric infections, as well to test novel mucosal vaccines and therapeutic approaches. Many past studies have been hampered by the fact that most captive macaques in existing colonies have been exposed to infectious agents. In colonies of captive *M. mulatta*, recurring diarrhea (chronic enterocolitis) is the leading cause of animal morbidity [24–26]. Chronic enterocolitis and diarrhea are associated with a variety of enteric pathogens including *C. coli*, *C. jejuni*, *Shigella flexneri*, *Yersinia enterocolitica*, adenovirus, and *Strongyloides fulleborni* [27].

Campylobacter spp. can be detected in feces of monkeys of which are clinically healthy, with diarrhea, and dead from acute enteric infections. The presence of *C. jejuni* as the only pathogen in some monkeys with diarrhea has led to its incrimination as the etiological agent in some episodes of acute enteric infection [28]. However, whereas *C. jejuni* has been recovered from 70% of monkeys with acute enteric infections, it was also found in 51% of healthy monkeys.

When feces from normal and diarrheic animals have been cultured for *C. jejuni* [29], a clear difference could not be detected in the rate of carriage between normal versus diarrheic cattle, horses, pigs, and dogs. The biotypes of *C. jejuni* recovered were the same as those associated with *Campylobacter*-induced enteritis in human beings [30]. Intestinal carriage rates for *Campylobacter* spp. between dogs with gastroenteritis and healthy controls were compared [31]. For dogs older than 12 months, there was no difference in *Campylobacter* carriage rates between diarrheic and healthy animals. However, in younger dogs 44% of animals

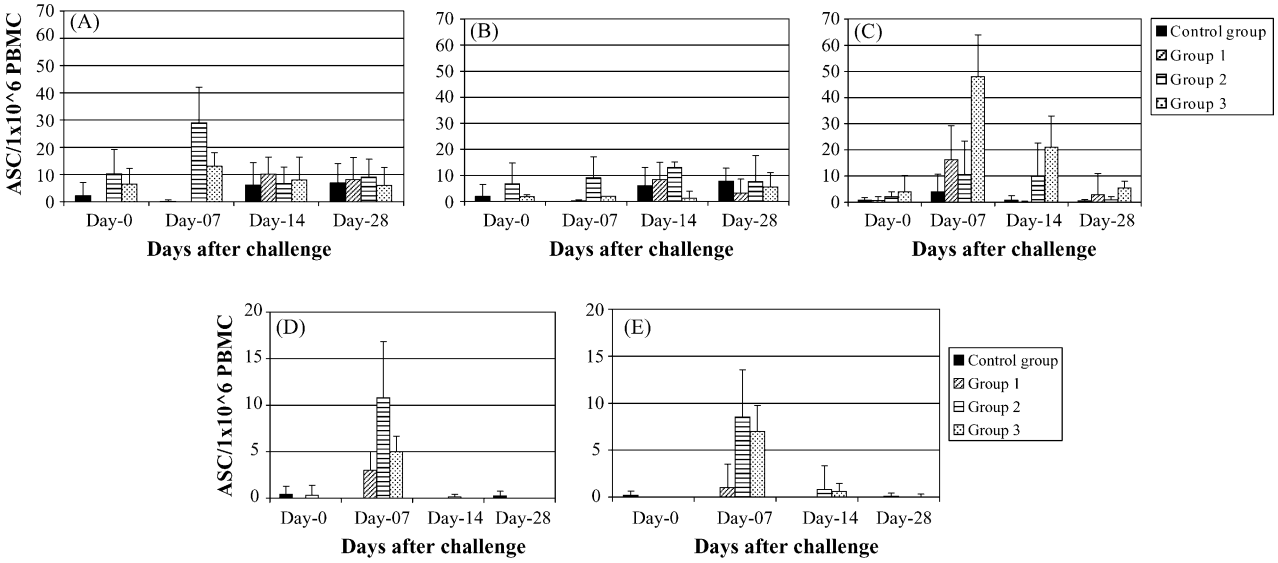

Fig. 3. Mean total IgA (A), IgG (B), and IgM (C) ASC in control group and three challenged group, detected by ELIPOT assay. F-CJ 81-176 strain specific IgM ASC in control group and three challenged monkey groups (D), and GE specific IgM ASC (E).

with diarrhea shed *Campylobacter* in their feces, more than twice the rate in asymptomatic controls (21%).

Campylobacter infections also can cause enteritis and abortions in cattle [32]. *Campylobacter* DNA was detected in 75% of the fecal samples from the feces of dairy cows in Alberta, Canada [33].

In our Veterinary Medicine Department at AFRIMS, we house circa 500 Rhesus monkeys. Recently, most of the monkeys from the colony where screened for serum IgA and IgG titers against *Campylobacter*. None of the monkeys had diarrhea during the screening period. However, 9% of the monkeys were found to be asymptomatic carriers of *C. jejuni*. Among asymptomatic carriers for *Campylobacter*, 30% of the monkeys had IgA titers >100. Among monkeys with no enteric pathogens in stool, 13% had IgA titer >100.

The objective of the present study was to establish a non-human adult primate model of *Campylobacter* infection that could allow evaluation of the safety, immunogenicity and efficacy of candidate *C. jejuni* vaccines. The expectation was that by feeding groups of adult rhesus monkeys 10^7, 10^9 or 10^{11} cfu strain 81-176 (known to be pathogenic for humans), it would be possible to identify a challenge inoculum that would cause moderate diarrhea in $\geq$80% of monkeys. Unfortunately, this attack rate was not achieved even with the highest dose (although monkeys did experience mild disease).

Investigators have previously tried with small animal models (mice) to study immune responses to *Campylobacter* infection. An intranasal mice model was used for the study of *Campylobacter* pathogenesis and immunity [16,17]. Mice were challenged with a dose of 5×10^9 cfu, and within 4 h after challenge *C. jejuni* were isolated from different organs. The intranasal mouse model was shown as a useful model for the study of infection.

It has been reported that young Rhesus macaque monkeys can be infected with a *C. jejuni* causing bacteremia [13]. Recovered animals were protected against re-infection, which suggests that initial experimental infection induces a strong immunity in those young monkeys. This could be the reason why our adult rhesus monkeys did not develop notable illness when these monkeys were challenged with high doses of a *C. jejuni* pathogenic strain. However, the monkeys that we challenged had low pre-challenged IgA and IgG titers. Although there was no diarrhea in the challenged monkeys, the elicited immune responses were high and dose-dependent. There were no *C. jejuni*-specific IgA or IgG ASC responses recorded against *Campylobacter* antigens but IgM-ASC responses were observed. One interpretation of these ASC data (albeit unlikely), is that these monkeys were encountering *Campylobacter* infection for the first time.

The results from this study indicate that adult Rhesus monkeys may not be the choice for experimental infection with pathogenic *Campylobacter* spp, although they may serve as a model for measuring immune responses to vaccines.

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